

## *Nuphar submersa* (Nymphaeaceae), a New Species from Central Japan

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A new species of *Nuphar* (Nymphaeaceae), *N. submersa*, from Tochigi Prefecture, central Japan, is described. *Nuphar submersa* is characterized by its submerged habit and narrowly oblong-triangular leaves that lack a sinus. It differs from both *N. japonica* and *N. oguraensis* in the color of the anthers and fruits. A phylogenetic study based on Amplified Fragment Length Polymorphism (AFLP) fragments showed that both *N. submersa* and *N. oguraensis* were sister taxa, but they were well separated phylogenetically.

Key words: AFLP, central Japan, new species, *Nuphar submersa*, phylogeny, submerged plant, Tochigi Prefecture

*Nuphar* Sm. (Nymphaeaceae), yellow water-lily, is a perennial freshwater macrophyte of the temperate Northern Hemisphere. According to Kitamura & Murata (1961), Ohwi (1965), Tamura (1982) and Kadono (1994), four species with one to four varieties or forms have been recognized in Japan; *Nuphar japonica* DC., *N. oguraensis* Miki, *N. subintegerrima* (Casp.) Makino and *N. pumila* (Timm) DC. The former three species are restricted to eastern Asia and the last species is distributed widely in the Old World (Hara 1951, Beal 1956, Ohwi 1965, Tamura 1982, Kadono 1994).

Plants of *Nuphar* known from Tochigi Pref. with narrowly oblong-triangular submerged leaves and red stigmatic discs have been identified as either *N. japonica* or *N. subintegerrima* (Sekimoto

1951, Plant Society of Tochigi Prefecture 1968, Hasegawa 1982, Botany Section of Natural Environment Research Group of Tochigi Prefecture 2003a, b), but in 2001 Hasegawa reported the plants to be different from other species known from Japan. The Tochigi plants have submerged leaves and Shiga *et al.* (2005) believe them to be similar to *N. oguraensis* in several morphological features.

Our detailed morphological and molecular comparison of the Tochigi plants with related species revealed that they should be treated as a new species.

***Nuphar submersa* Shiga & Kadono, sp. nov.** (Figs. 1 & 2).

*Nuphar* sp., Plants of Utsunomiya City 146,

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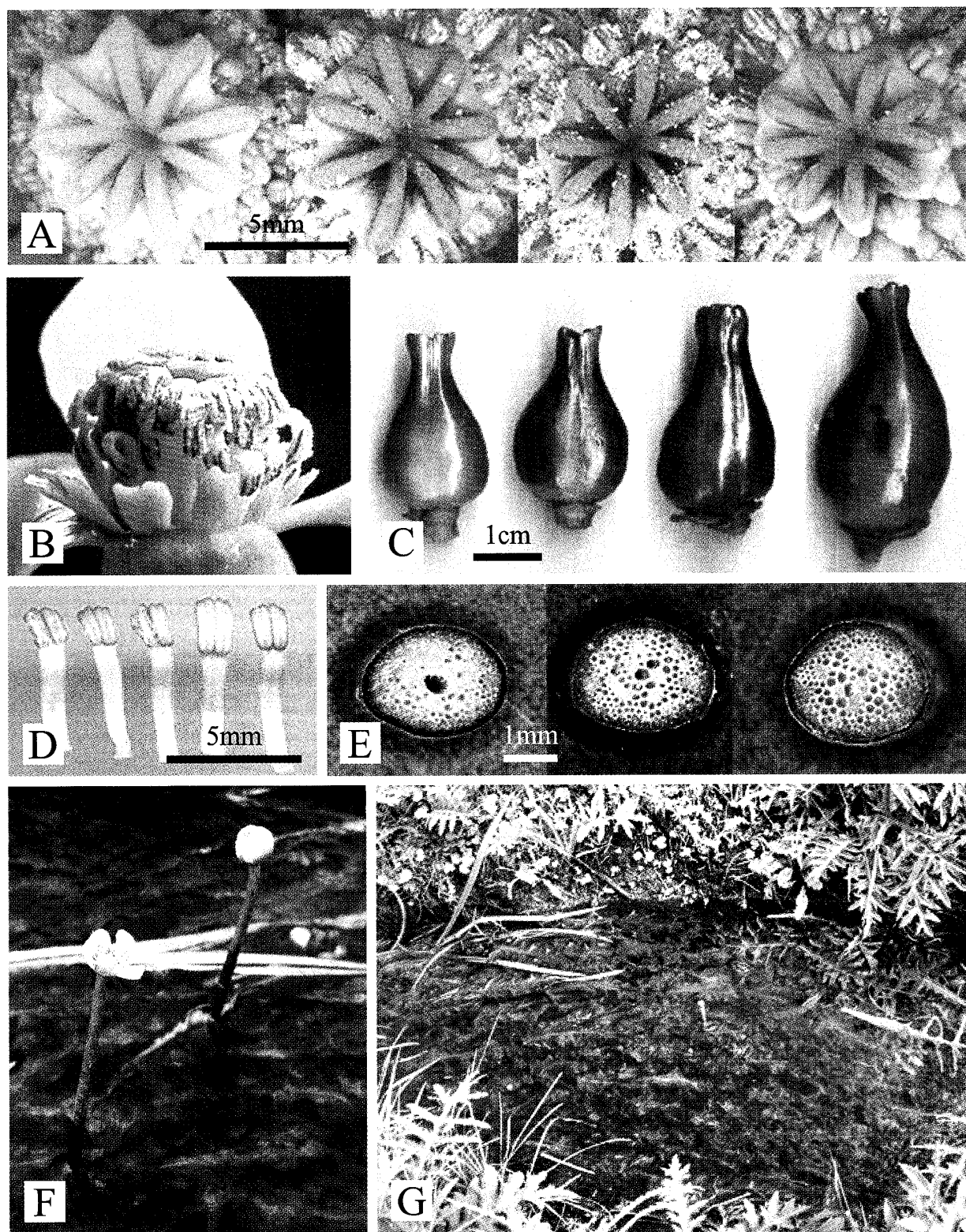


FIG. 1. *Nuphar submersa* Shiga & Kadono, sp. nov. from TG1. A: Stigmatic disc. B: Flower. C: Fruit. D: Stamen. E: Transverse section of petiole. F-G: Habit.

tab. 34 (2001).

*Nuphar oguraensis* Miki var. *submersa* Shiga

& Kadono in Bull. Water Pl. Soc. Japan 82: 47 (2005), nom. nud.

TABLE 1. Localities of 29 samples of *Nuphar* and *Barclaya*. Herbarium abbreviations in parentheses.

Pop. Code	Sampling locality	Latitude (N)	Longitude (E)	Voucher for DNA
<b><i>N. submersa</i></b>				
TG1	Tochigi Pref.: Koshiro, Nikko-shi	36 ° 39'	139 ° 43'	<i>T. Shiga 3480</i> [KYO, OSA, TNS]
TG2	Tochigi Pref.: Shimoikawa, Nasukarasuyama-shi	36 ° 42'	140 ° 6'	<i>H. Hirayama s.n.</i> [KOBE]
<b><i>Nuphar japonica</i></b>				
YA1	Yamagata Pref.: Tazawa, Murayama-shi	38 ° 32'39"	140 ° 22'12"	<i>T. Shiga 3120</i> [KOBE, OSA]
MY6	Miyazaki Pref.: Toyomitsu-cho, Miyakonojo-shi	31 ° 41'23"	131 ° 5'47"	<i>T. Shiga 3279</i> [KOBE, OSA]
<b><i>N. lutea</i></b>				
	SLOVAKIA: Podunajská Rovina: Klúčovec, Zátonoský les	47 ° 47'00"	17 ° 41'44"	<i>H. Kudoh et al. 03-507</i> [OSA]
<b><i>N. oguraensis</i> var. <i>oguraensis</i></b>				
GI4	Gifu Pref.: Hora, Gifu-shi	35 ° 28'32"	136 ° 43'5"	<i>T. Shiga 3356</i> [OSA]
HY8	Hyogo Pref.: Namita, Sanda-shi	34 ° 57'50"	135 ° 9'47"	<i>T. Shiga 3149</i> [KOBE, OSA]
HI7	Hiroshima Pref.: Oguni, Seranishi-cho	34 ° 38'2"	132 ° 55'50"	<i>T. Shiga 3240</i> [KOBE, OSA]
FO1	Fukuoka Pref.: Ikenoyama, Hoshino-mura	33 ° 14'47"	130 ° 45'21"	<i>T. Shiga 3666</i> [KOBE, OSA]
MY2	Miyazaki Pref.: Nagai, Kitagawa-cho	32 ° 40'11"	131 ° 43'17"	<i>T. Shiga 3314</i> [KOBE, OSA]
<b><i>N. oguraensis</i> var. <i>akiensis</i></b>				
HHS	Hiroshima Pref.: Shitami, Higashihiroshima-shi	34 ° 24'30"	132 ° 43'9"	<i>T. Shiga 3244</i> [KOBE, OSA]
KO1	Kochi Pref.: Kohda, Kochi-shi	33 ° 32'30"	133 ° 31'5"	<i>T. Shiga 3364</i> [KOBE, OSA]
KO2	Kochi Pref.: Hata, Ino-cho	33 ° 31'34"	133 ° 26'15"	<i>T. Shiga 3362</i> [KOBE, OSA]
<b><i>N. pumila</i> var. <i>pumila</i></b>				
WAM	Hokkaido Pref.: Keihoku, Wakkanai-shi	45 ° 24'03"	141 ° 49'01"	<i>T. Shiga 3488</i> [KOBE, OSA]
KUT	Hokkaido Pref.: Takkobu, Kushiro-shi	43 ° 6'18"	144 ° 29'07"	<i>T. Shiga 3466</i> [KOBE, OSA]
URU	Hokkaido Pref.: Uryu-numa Moor, Uryu-cho	43 ° 42'00"	141 ° 36'06"	<i>T. Shiga 3678</i> [OSA]
TSC	Hokkaido Pref.: Numasawa., Tsubetsu-cho	43 ° 38'08"	143 ° 52'38"	<i>T. Shiga 3597</i> [OSA]
BEB	Hokkaido Pref.: Honbakkai, Betsukai-cho	43 ° 25'26"	145 ° 14'56"	<i>T. Shiga &amp; S. Takebayashi 73</i> [KOBE, OSA]
NEN	Hokkaido Pref.: Katsuragi, Nemuro-shi	43 ° 19'11"	145 ° 37'02"	<i>T. Shiga &amp; S. Takebayashi 55</i> [KOBE, OSA]
HAK	Hokkaido Pref.: Hamanaka, Hamanaka-cho	43 ° 06'37"	145 ° 06'09"	<i>T. Shiga &amp; S. Takebayashi 211</i> [KOBE, OSA]
KWS	Hokkaido Pref.: Shinsen-numa Moor, Kyowa-cho	42 ° 54'13"	140 ° 35'27"	<i>T. Shiga 3599</i> [OSA]
ATO	Hokkaido Pref.: Koinuma, Atsuma-cho	42 ° 38'11"	141 ° 53'39"	<i>T. Shiga 3447</i> [KOBE, OSA]
<b><i>N. pumila</i> var. <i>ozeensis</i></b>				
URU	Hokkaido Pref.: Uryu-numa Moor, Uryu-cho	43 ° 42'00"	141 ° 36'06"	<i>T. Shiga 3679</i> [OSA]
GM1	Gunma Pref.: Kamitashiro, Katashina-mura	36 ° 55'11"	139 ° 12'12"	Cultivated at Kobe Univ.
<b><i>N. pumila</i> var. <i>ozeensis</i> f. <i>rubroovaria</i></b>				
URU	Hokkaido Pref.: Uryu-numa Moor, Uryu-cho	43 ° 42'00"	141 ° 36'06"	<i>T. Shiga 3477</i> [OSA]
<b><i>N. shimadae</i></b>				
	Taiwan: Xinzhu, Taoyuan	25 ° 03'	121 ° 13'	Cultivated at Kobe Univ.
<b><i>N. subintegerrima</i></b>				
GI1	Gifu Pref.: Tachibokubora, Gifu-shi	35 ° 25'41"	136 ° 47'45"	<i>T. Shiga 3228</i> [KOBE]
MI1	Mie Pref.: Oshihuchi, Minamiise-cho	34 ° 19'36"	136 ° 37'58"	<i>T. Shiga 3234</i> [KOBE]
<b><i>Barclaya longifolia</i></b>				
	East Asia: Garden plants obtained from garden center			

“*Nuphar subintegerrima*” auct. non Makino: (1951); Plant Society of Tochigi Prefecture, List of Plants in Tochigi Prefecture 126 (1968); Sekimoto, Index of Plant of Tochigi Prefecture 19

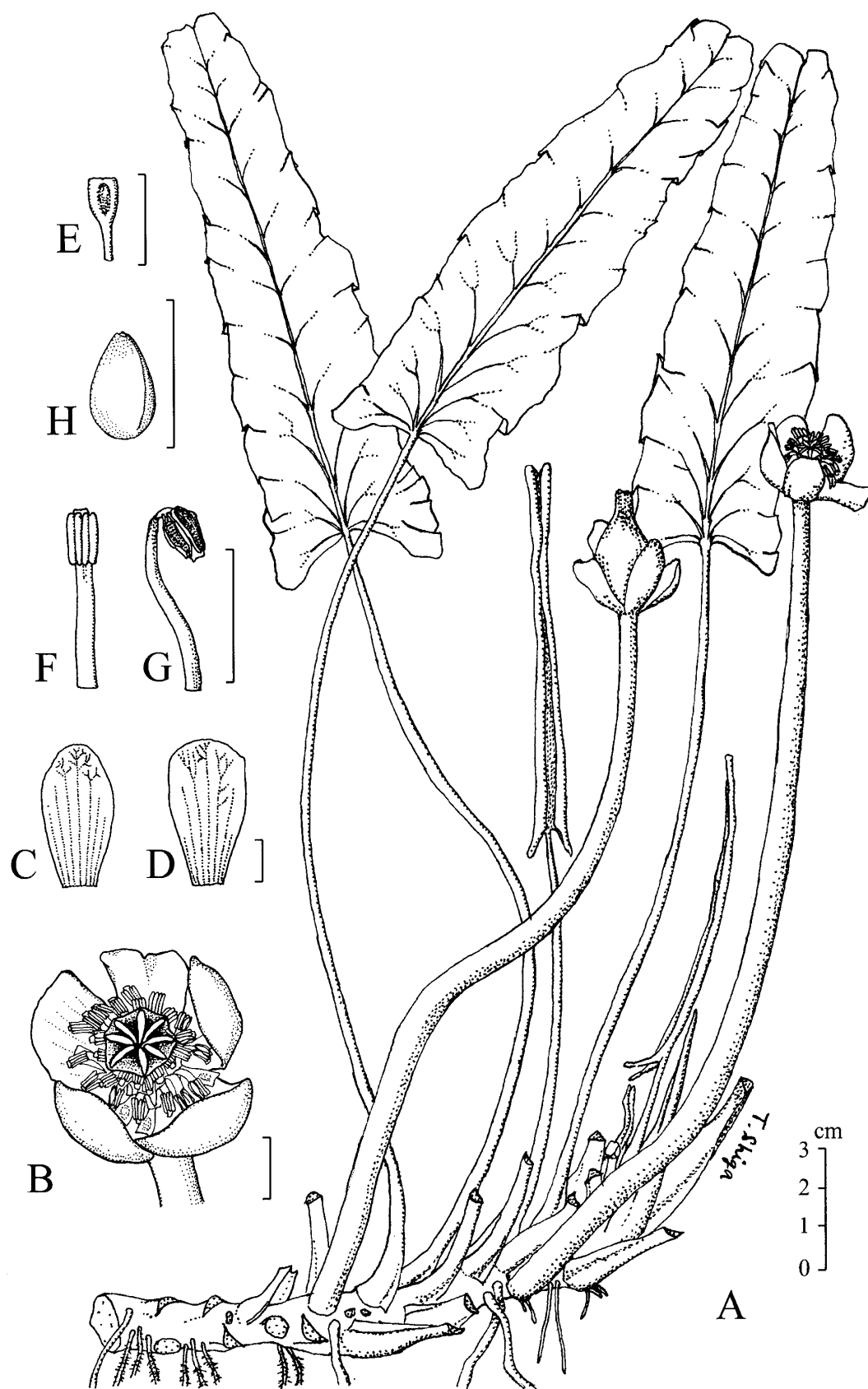


FIG. 2. *Nuphar submersa*. A: Whole plant. B: Flower. C-D: Sepal. E: Petal. F: Stamen. G: Stamen after anthesis. H: seed. A, F-G from *T. Shiga* 3480 (OSA). B-E, H from *T. Shiga* 3479 (OSA). Scale indicates 5 mm except A.

Hasegawa, Vegetation and Flora of Tochigi Prefecture, tab. 155 (1983); Komine, Plants of Tochigi 1: 199 (2003).

“*Nuphar japonica*” auct. non DC.: Komine, Plants of Tochigi 1: 199 (2003), p. p.; Komine, Plants of Tochigi 2: 142 (2003), p. p.

*Nuphar japonicae* DC. et *N. oguraensi* Miki proxima, sed in foliis submersis anguste oblongis triangularis, non sinuosis, antheris rufescentibus et fructibus rubris differt.

*Typus*: JAPAN; Tochigi Pref., Imaichi-shi, Koshiro, alt. 240 m, 29 Sep 2004, *T. Shiga* 3480 (Holotype OSA; Isotypes KYO, TNS).

Perennial aquatic herbs. Rhizomes slender, procumbent, branching. Leaves submerged or rarely floating in lentic water; submerged leaves narrowly oblong-triangular, 10-18 cm long, 2-5 cm wide (Fig. 2), membranaceous, undulate, without sinus; floating leaves narrowly ovate, base slightly cordate-sagittate. Petiole flattened, usually with central lacuna (Fig. 1E). Peduncle emergent. Flowers June to October, 2-3 cm across, protogynous; sepals 5, obovate, yellow, apex rounded, 1-2 cm long (Figs. 2C-D); petals spatulate, yellow, 5-7 mm long (Fig. 2E); anthers strongly recurved after anthesis (Figs. 1B & 2G), 1.5-2.5 mm long, anther to filament ratio 1:2 to 1:3, pollen sack tinged orange or red (Figs. 1D & 2F); carpels many, fused; stigmatic disc tinged reddish or dark red, 4-8 mm across, rays 7-9, shallowly dentate, stigmatic rays usually regularly arranged, 2-3 mm long (Fig. 1A); fruit reddish, ovoid, 2-3 cm long (Fig. 1C); seeds numerous, narrowly ovoid to ovoid, 3.5-4.5 mm long, 2.5-3.5 mm wide (Fig. 2H).

*Japanese name*: Shimotsuke-kōhone (Shiga and Kadono 2005)

*Distribution*: Japan (Tochigi Pref.). Endemic.

*Habitat*: Rivers and streams.

*Other specimens examined*: **JAPAN: Tochigi Pref.**, Hosyakuji-mura, Jul. 31, 1951, *Y. Matsumura* s.n. (TNS 011391, 016444); Mine-cho, Utsunomiya-shi, Sept. 18,

1947 (TOCH 121889); Shimokawai, Minaminasu-machi, alt. 130 m, Sept. 6, 1995, *Y. Komine* 81551 (TOCH); Nov. 2004, *H. Hirayama* s.n. (KOBE); Aug. 6, 2005, *T. Shiga* 3560-3562 (KOBE, OSA); Koshiro, Imaichi-shi, alt. 240 m, Jul. 26, 2003, *M. Komakura & T. Sugawara* s.n. (TOCH 139148); Sept. 30, 2004, *T. Shiga* 3479 (KOBE, OSA), Aug. 6, 2005, *T. Shiga* 3595 (KOBE); Sakazura, Kawachi-cho, Kawachi-gun, Aug. 17, 1980, *T. Noguchi* s.n. (KOBE); Nomoto-gawa River, Higashimizu-numa, Haga-cho, Haga-gun, Aug. 8, 1988, *Y. Kadono* 5528 (KOBE).

## Materials and Methods

### Plant materials

Twenty eight plants of *Nuphar* were collected from 26 localities in Japan, Taiwan and Slovakia (Table 1). The European *N. lutea* (L.) Sm. was added to compare with the Asian taxa. Based on the results of a molecular phylogenetic study by Les *et al.* (1999), *Barclaya longifolia* Wall. was chosen as the out-group taxon in the phylogenetic study.

For morphological analysis of *Nuphar submersa*, more than ten submerged leaves, flowers and fruits were collected from two populations (TG1 & TG2; Table 1). All measurements were made using material fixed in FAA (ethanol - formalin - acetic acid) in the field. Specimens of *N. submersa* in the herbarium of the National Science Museum, Tokyo (TNS), Osaka Museum of Natural History (OSA), Tochigi Prefectural Museum (TOCH) and Kobe University (tentatively abbreviated KOBE) were also examined.

### Amplified Fragment Length Polymorphism (AFLP) analysis

Total DNA was extracted from 50-100 mg dry mass of leaves of each accession by the CTAB method (Stewart & Via 1993) with some modifications and approximately 200 ng of total DNA per sample was obtained.

Amplified Fragment Length Polymorphism (AFLP) analysis was performed on all 29 samples according to the method of Vos *et al.* (1995) with

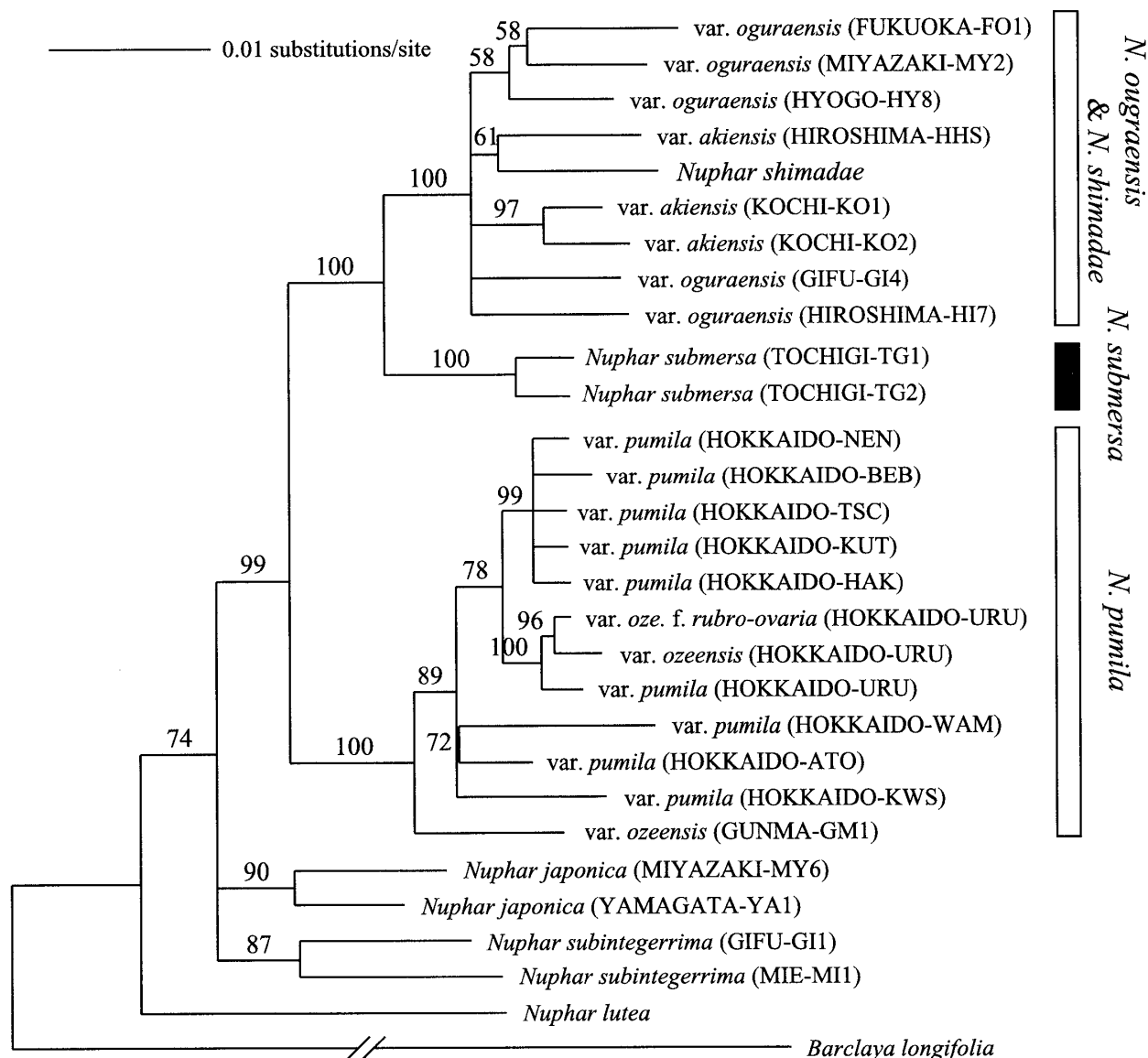


FIG. 3. Neighbor-joining tree based on 788 AFLP fragments. Bootstrap values (>50%) based on 1000 replicates are shown above branches. Sampling locality and population code are shown in parentheses.

some modifications. Sample DNA was restricted with the endonucleases *Eco*RI and *Mse*I at 37°C in 1.5 hours and ligated to appropriate double-stranded adapters at 20°C overnight. Two steps of amplification followed; a preselective amplification in which we used primers with a one base pair (bp) extension, and a second amplification in which primers with 3 bp extensions were used, thereby further reducing the number of fragments. For the second amplification, we initially tried 16 different primer combinations. From them, we selected four

combinations, -ACA(FAM)/-CTA, -ACG(FAM)/-CTA, -ACA(JOE)/-CTG and -ACG(JOE)/-CTG, for the extensions to the *Eco*RI and *Mse*I sites, respectively.

The PCR products from the selective amplification were visualized using an ABI Prism 3100 automated sequencer. Fragments from 50 to 500 bp in size were sized with GeneScan 500 ROX Size Standard (Applied Biosystems).

Sizing and quantification of AFLP fragments with GeneScan 3.7 (Applied Biosystems) and the

TABLE 2. Comparison of diagnostic characters in *Nuphar submersa*, *N. oguraensis*, *N. pumila* and *N. japonica*. Data for *N. japonica*, *N. oguraensis*, *N. pumila* and *N. subintegerrima* are from Kadono (1994), Shiga & Kadono (2004) and Takahashi *et al.* (2005)

	<i>N. submersa</i>	<i>N. japonica</i>	<i>N. oguraensis</i>	<i>N. pumila</i>	<i>N. subintegerrima</i>
Life form	submerged	emergent	floating-leaved	floating-leaved	emergent or floating-leaved
Emergent or floating leaf	narrowly ovate*	narrowly ovate	widely ovate to ovate	widely ovate to ovate	roundish
Submerged leaf	narrowly oblong-triangular	narrowly ovate to ovate	roundish to ovate	roundish to ovate	roundish to ovate
Sinus of submerged leaf	shallow or absent	deep	deep	deep	deep
Central lacuna of petiole	usually present	absent	present	absent	absent
Stigmatic disc color	red	yellow	red or yellow	red or yellow	orange or yellow
Anther and filament length ratio	1:2~1:3	1:1~1:2	1:2~1:3	1:2~1:3	1:1
Anther color	red	yellow	yellow	yellow	yellow
Fruit color	red	green	green	red or green	red or green

\*Leaves of *Nuphar submersa* are always submerged in natural populations. They are rarely floating in lentic water.

electropherograms were imported into Genotyper 3.7 (Applied Biosystems). Assignment of AFLP fragments of size categories was performed by manual evaluation of all electropherograms using Genotyper 3.7. The AFLP fragments were aligned after size categories in a data matrix and either a value 0 or 1 (0 = fragment absence; 1 = fragment presence) was assigned.

#### Data analysis

To evaluate genetic distance, the proportion of shared fragments for each pair of samples ( $F$ ; Nei & Li 1979, Innan *et al.* 1999) was calculated from the AFLP data. From the value of  $F$  obtained for each taxon, we estimated the nucleotide diversity ( $\pi$  or  $d$ ), i.e., the average number of nucleotide substitutions per nucleotide site, using the method of

Innan *et al.* (1999) for AFLP data. Phylogenetic analysis was performed using the NEIGHBOR program from the PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein 1993). A phylogenetic tree based on nucleotide diversity ( $d$ ; Nei & Miller 1990, Innan *et al.* 1999) as a measure of genetic distance was constructed using neighbor-joining method.

## Results and Discussion

#### Morphological characters

*Nuphar submersa*, from Tochigi Prefecture, is a mostly submerged plant inhabiting rivers and streams (Figs. 1F-G). It resembles *N. japonica* in the morphology of the submerged leaves and *N. oguraensis* in flower morphology, anther to filament

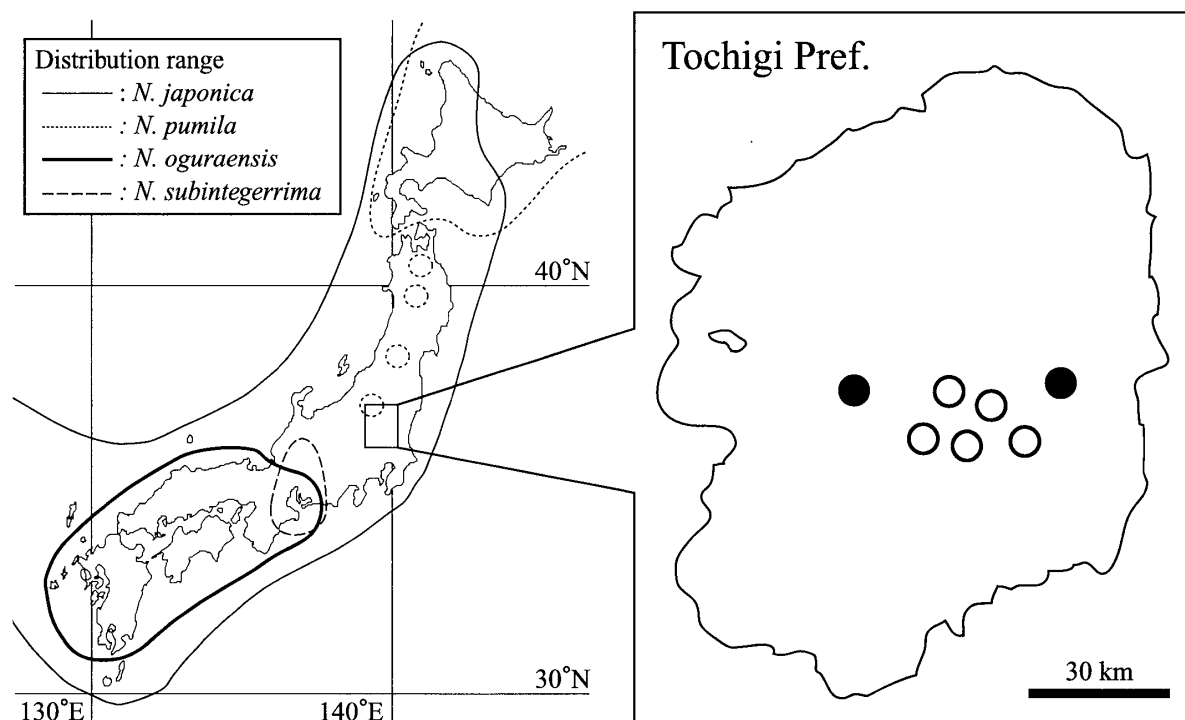


FIG. 4. Distribution of *Nuphar submersa* based on herbarium specimens. Symbols indicate herbarium specimen of extant (●) and extinct (○) populations. Distribution ranges of the four other species of Japanese *Nuphar* are also shown.

length ratio and petiole anatomy (Figs. 1A-D & 2). A comparison of the diagnostic characters of *N. submersa*, *N. japonica*, *N. oguraensis*, *N. pumila* and *N. subintegerrima* is summarized in Table 2. From our observations of fresh material and herbarium specimens, *N. submersa* has submerged leaves without a sinus at the base, which differs from the submerged leaves with a deep sinus in *N. japonica*. The petiole with a central lacuna resembles that of *N. oguraensis*, but differs from that of *N. pumila*, although the anther to filament length ratio is similar to that of both *N. oguraensis* and *N. pumila*. The reddish anthers and fruit of *N. submersa* (Figs. 1C-D) differ from the yellow anthers and green fruit of *N. japonica* and *N. oguraensis*. Based on the above observations, we concluded that the plants from Tochigi Pref. differed from all other species.

When *Nuphar submersa* was cultivated in lentic water, the plants rarely formed incomplete floating leaves with a partially cuticularized leaf blade (Shiga & Kadono unpublished data, Komine

personal communications). It is probable that *N. submersa* lost the genetic ability to form floating leaves. All taxa of the genus *Nuphar* have been reported to be emergent or to have floating leaves (cf. Beal 1956, Padgett *et al.* 1999, Padgett 2003). Further ecological study of *N. submersa* is needed to determine the evolutionary nature of its life form.

#### AFLP analysis

A total of 788 different AFLP size categories of fragments was observed from four selective primer pairs. For each of the primer pairs 7-37 (3.4-22.0%) of the total 176-216 fragments scored was unique for the outgroup taxon. Of the total 714 fragments scored for the species of *Nuphar*, 684 fragments (95.8%) were polymorphic. For each of the four Japanese species (*N. japonica*, *N. oguraensis*, *N. pumila* and *N. subintegerrima*), 194-240 fragments were scored per sample on average. For *N. submersa*, 202 (TG1) and 216 (TG2) fragments were scored. Thirteen fragments were fixed in TG1 and



TG2 as species-specific fragments of *N. submersa*. All 29 plants examined were revealed to have unique genotypes.

The neighbor-joining tree showed a sister group relationship between *Nuphar submersa* and *N. oguraensis*, including *N. shimadae* from Taiwan (Fig. 3), with 100% bootstrap support. Japanese and Taiwanese plants of *Nuphar* were monophyletic with a 74% bootstrap value (Fig. 3). Within the group, four main monophyletic groups were recognized; the first cluster consisted of *N. oguraensis*, *N. shimadae* and *N. submersa*; the other three clusters corresponded to *N. pumila*, *N. japonica* and *N. subintegerrima*, respectively. These four clusters were strongly supported with high bootstrap values (87-100%). In terms of the relationship among the four main monophyletic groups, *N. pumila* and *N. oguraensis*, including *N. shimadae* and *N. submersa*, formed sister groups with 99% bootstrap support, although the relationship between *N. japonica* and *N. subintegerrima* was poorly resolved. In the *N. oguraensis* group, two subclusters were recognized. The first subcluster of two OTUs consisted of *N. submersa* and *N. oguraensis* and *N. shimadae* complex, which are morphologically similar (Miki 1934), with 100% support. It is probable that *N. oguraensis* and *N. shimadae* are conspecific.

The neighbor-joining topology supported this interspecific relationship, which was proposed by previous phylogenetic studies based on morphology and ITS and *matK* DNA sequences (Padgett *et al.* 1999).

The phylogenetic study showed both *Nuphar submersa* and *N. oguraensis* to be sister taxa, but well separated phylogenetically. The reddish anthers and fruit and habit of *N. submersa* with mostly submerged leaves alone separate it from *N. oguraensis*. *Nuphar submersa* is also located ca. 300 km east of the easternmost locality of *N. oguraensis* in Aichi Prefecture (Fig. 4).

Examination of herbarium specimens showed that *N. submersa* is restricted to Tochigi Prefecture (Fig. 4), where only two small populations were located during our field work. Most of the populations are small; the population size of TG1 was 0.5 m × 30 m and that of TG2 occurred fragmented within a range of 1 m × 100 m. The species is in urgent need of protection.

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### Key to the Japanese species of *Nuphar*

- A. Leaves always submerged under natural conditions; submerged leaves narrowly oblong-triangular, base slightly cordate-sagittate ..... *N. submersa*
- A. Leaves emergent, floating or submerged; submerged leaves narrowly ovate to rounded, base with deep sinus
  - B. Emergent leaves narrowly ovate, 25-50 cm long; submerged leaves narrowly ovate to ovate ..... *N. japonica*
  - B. Emergent and floating leaves ovate to rounded, 4-22 cm long; submerged leaves ovate to rounded
    - C. Anther to filament length ration 1:1; seeds 5.5-6.5 mm long ..... *N. subintegerrima*
    - C. Anther to filament length ration 1:2 to 1:3; seeds 3-5 mm long
      - D. Petiole with central lacuna ..... *N. oguraensis*
      - D. Petiole solid ..... *N. pumila*

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